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Relative Importance of Heat-Labile Enterotoxin in the Causation of Severe Diarrheal Disease in the Gnotobiotic Piglet Model by a Strain of Enterotoxigenic *Escherichia coli* That Produces Multiple Enterotoxins†

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Enterotoxigenic *Escherichia coli* (ETEC) strains that produce multiple enterotoxins are important causes of severe dehydrating diarrhea in human beings and animals, but the relative importance of these enterotoxins in the pathogenesis is poorly understood. Gnotobiotic piglets were used to study the importance of heat-labile enterotoxin (LT) in infection with an ETEC strain that produces multiple enterotoxins. LT$^-$ (Δ*eltAB*) and complemented mutants of an F4$^+$ LT$^+$ STb$^+$ EAST1$^+$ ETEC strain were constructed, and the virulence of these strains was compared in gnotobiotic piglets expressing receptors for F4$^+$ fimbria. Sixty percent of the piglets inoculated with the LT$^-$ mutant developed severe dehydrating diarrhea and septicemia compared to 100% of those inoculated with the nalidixic acid-resistant (Nal^r) parent and 100% of those inoculated with the complemented mutant strain. Compared to piglets inoculated with the Nal^r parent, the mean rate of weight loss (percent per hour) of those inoculated with the LT$^-$ mutant was 67% lower ($P < 0.05$) and that of those inoculated with the complemented strain was 36% higher ($P < 0.001$). Similarly, piglets inoculated with the LT$^-$ mutant had significant reductions in the mean bacterial colony count (CFU per gram) in the ileum; bacterial colonization scores (square millimeters) in the jejunum and ileum; and clinical pathology parameters of dehydration, electrolyte imbalance, and metabolic acidosis ($P < 0.05$). These results indicate the significance of LT to the development of severe dehydrating diarrhea and postdiarrheal septicemia in ETEC infections of swine and demonstrate that EAST1, LT, and STb may be concurrently expressed by porcine ETEC strains.

Enterotoxigenic *Escherichia coli* (ETEC) is an important cause of diarrhea and death in human beings and animals (42). ETEC is thought to cause diarrhea predominantly through the production of enterotoxins, which induce water and electrolyte loss from the intestine (42). ETEC strains are known to produce several types of enterotoxins, including heat-labile enterotoxin (LT), heat-stable enterotoxin a (STa), STb (42), and enteroaggregative *E. coli* heat-stable enterotoxin 1 (EAST1) (28, 51, 67). An individual ETEC strain may produce one or more enterotoxins (28, 36, 41, 66); however, ETEC must also produce fimbriae and in some cases must infect a host that expresses the corresponding fimbrial receptors in order to cause severe dehydrating diarrheal disease (16, 33, 55).

In swine, the most common and severe ETEC infections are caused by strains that express K88 (F4) fimbriae (33). Piglet enterocyte susceptibility to F4$^+$ ETEC adherence is correlated with expression of an intestinal mucin-type glycoprotein (IMTGP) receptor for the F4$^+$ fimbria (16, 20). The increased virulence of F4$^+$ ETEC strains in susceptible swine is evidenced clinically by their tendency to cause extensive intestinal colonization, severe dehydrating diarrhea, postdiarrheal septicemia, and death (13, 14, 30, 40, 41). The increased virulence of these strains in susceptible swine is due in part to their ability to colonize the entire small intestine instead of only the ileum, as occurs with K99 (F5$^+$), 987P (F6$^+$), and F41$^+$ strains (3, 24). The pathogenesis of postdiarrheal septicemia is poorly understood but is related to the development of severe dehydration, hypovolemic shock, and ischemia of the intestinal mucosa, the last presumably a consequence of the shock-induced low-flow state (6, 22, 40). Histological examination of immunohistochemically stained small intestinal tissue sections of moribund or dead piglets in cases of natural and experimental infection reveal ETEC bacteria adherent to exposed intestinal basement membranes and within juxtaposed villous capillaries (40). Based on these observations, we hypothesized that severe dehydration causes hypovolemic shock and ischemic bowel necrosis and that the latter predisposes the piglet to postdiarrheal septicemia via translocation across exposed intestinal basement membranes.

F4$^+$ ETEC isolates from swine commonly produce both LT and STb (36, 41, 66), are often PCR positive for the EAST1 gene (7, 68), and frequently cause death in natural infections (40, 41). To our knowledge, EAST1 expression by porcine

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TABLE 1. *E. coli* strains and plasmids used

Strain or plasmid	Relevant properties	Vector	Source or reference
E. coli strains			
17-2	Enteroaggregative *E. coli*, wild type		50
2534-86	O8:K87:NM:F4ac LT-I$^+$ STb$^+$, wild type		40
S17-1λ*pir*	RP4-2-Tc::Mu Km::Tn*7 pro res* Mod$^+$		31, 53
WAM2317	Nal^r mutant of 2534-86		40
MUN283	S17-1λ*pir*(pMUN283), LT-I$^-$ Km^r Ap^r		This study
MUN284	Nal^r Km^r, LT-I$^-$ mutant of WAM2317		This study
MUN285	Nal^r Km^r, LT-I$^-$ mutant of WAM2317		This study
MUN287	Nal^r Km^r, LT-I$^+$ complemented strain		This study
G58-1	Nonpathogenic, wild type, LT-I$^-$		5
DH5α	K-12 laboratory strain		1
BF3	DH5α(pKK223-3/*astA*), EAST1$^+$		17
CB2091	K-12 (pWD600), LT-I$^+$ Tc^r		M. Bagdasarian
NADC 2290	K-12 strain HB101		1
NADC 2329	HB101(pRAS1), STb$^+$		4, 65
NADC 2787	K-12(pBR322), STb$^-$		T. Casey
NADC 4924	HB101(pRAS1 Δ*Bgl*II)		T. Casey
Plasmids			
pCVD442	MobRP4$^+$ R6K$^+$ SacB$^+$ Ap^r	pGP704	10
pKK223-3	Ap^r	pBR322	Amersham
pMMB66	Ap^r		38
pMUN281	LT-I$^-$ Km^r	pWD600	This study
pMUN283	LT-I$^-$ Km^r Ap^r	pCVD442	This study
pMUN287	LT-I$^+$	pMMB66	This study
pRASI	STb$^+$	pBR322	4, 65
pUC4K	Km^r Ap^r	pUC7	59
pWD600	LT-I$^+$ Tc^r	pBR322	9

ETEC isolates has not been reported in the literature and consequently nothing is known of the significance of this enterotoxin, if any, in porcine ETEC infections. The ability to produce multiple enterotoxins is a rational hypothesis for explaining why some ETEC strains are more virulent. However, there is a lack of information concerning the contribution of the different enterotoxins to virulence, especially in light of defined fimbrial type and host susceptibility.

In the present study, the objective was to test the significance of LT for induction of severe dehydrating diarrhea and postdiarrheal septicemia in F4$^+$ LT$^+$ STb$^+$ ETEC infection of piglets. We were particularly interested in the contribution of LT in F4$^+$ LT$^+$ STb$^+$ strains that potentially also express EAST1, because these strains are both highly prevalent (7, 36, 41, 67, 68) and virulent (14, 30, 40) in swine. We hypothesized that inactivation of the LT-encoding *eltAB* genes in such a strain would reduce the development of severe dehydrating diarrhea, hypovolemic shock, and postdiarrheal septicemia in IMTGP$^+$ piglets. This hypothesis was based on the results of previous studies showing the highly toxic effects of crude LT (whole-cell lysates) in piglets (25) and an inability of STb to cause severe diarrhea in neonatal piglets (4). We found that piglets inoculated with an Δ*eltAB* mutant strain had a significantly reduced rate of development of severe dehydrating diarrhea and postdiarrheal septicemia, but each condition still occurred within 96 h postinoculation (p.i.). Piglets inoculated with the *eltAB* mutant strain had significantly less bacterial colonization of the small intestine than did piglets inoculated with the Nal^r parent and complemented strains. These results confirm the importance of LT in the pathogenicity of F4$^+$ LT$^+$ STb$^+$ ETEC strains and provide evidence that EAST1, LT, and STb may be concurrently expressed by porcine ETEC strains.

(A portion of this work was presented at the 101st General Meeting of the American Society for Microbiology, Orlando, Fla., 20 to 24 May 2001.)

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are shown in Table 1. Porcine ETEC strain 2534-86 and its spontaneous nalidixic acid resistant (Nal^r) mutant, WAM2317, were previously described (40). Strain CB2091, an *E. coli* K-12 strain transformed with plasmid pWD600, was kindly provided by M. Bagdasarian. Plasmid pWD600 contains *eltAB* within a PstI fragment, and the corresponding 0.5-kb upstream and 4.0-kb downstream regions (9). Suicide plasmid pCVD442 (10) and the corresponding host strain (S17-18λ*pir*) (31, 53) were kindly provided by R. Welch. STb$^+$ strain NADC 2329 (4, 65) and STb$^-$ strains NADC 2290 (1, 65), NADC 2787, and NADC 4924 were kindly provided by T. Casey. Wild-type enteroaggregative *E. coli* strain 17-2 (50) and EAST1 clone BF3 (17) were kindly provided by W. Fusco.

Plasmid and total genomic DNA isolation, recombinant DNA procedures, and Southern blots. Plasmid DNA was extracted from bacterial cultures by an alkaline lysis procedure (1). Proteins were initially removed by the addition of a potassium acetate solution and centrifugation. Plasmid DNA from ETEC strains was precipitated by the addition of isopropanol and further purified by an acid phenol extraction procedure for supercoiled DNA (62). The different plasmids from ETEC strains were isolated from 0.7% agarose gels and subjected to HindIII digestion. The sizes of the different plasmids in purified preparations were estimated from the sums of the sizes of the restriction fragments by using HindIII-digested lambda DNA as a molecular weight marker. In separate procedures, total genomic DNA was extracted from bacterial cultures by a lysozyme-cetyltrimethylammonium bromide lysis procedure (1). Recombinant DNA procedures and Southern blots were done as previously described (1, 48). Gene probes for Southern blots were directly labeled with horseradish peroxidase, and the hybridization products were detected with a chemiluminescence detection system (Amersham Pharmacia).

PCR amplification. Bacterial DNA to be amplified was isolated by a lysozyme-cetyltrimethylammonium bromide lysis procedure (1). PCRs were performed in a final volume of 100 μl containing 1× PCR buffer (Perkin-Elmer), 0.2 mM deoxynucleotide triphosphates, 1.5 mM $MgCl_2$, 0.5 μM each primer, and 2.5 U of AmpliTaq Gold polymerase (Perkin-Elmer). PCR conditions used for all primers included an initial cycle of denaturation at 92°C for 5 min, followed by 30 cycles of denaturation at 92°C for 1 min, annealing at 46°C for 1 min, and extension at 72°C for 1 min, with a final cycle of extension at 72°C for 5 min. The primers for PCR (forward primer, LTABU [5′-CGGATTGTCTTCTTGTATG AT]; reverse primer, LTABL [5′-GATCGGTATTGCCTCCTCTAC]) amplified a 1,274-bp fragment comprising the entire *eltAB* gene cluster. PCR products were analyzed by electrophoresis on 1% agarose gels, recovered with the Qiaquick gel extraction kit (QIAGEN), and characterized by restriction analysis with XbaI.

SDS-PAGE and immunoblot analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), urea-SDS-PAGE, and immunoblots were performed by standard methods (12, 48, 57). Protein extracts were standardized for total protein concentration. Rabbit anti-cholera toxin (anti-CT) (Sigma) and anti-STb sera (11) were used as primary antibodies for the LT and STb immunoblots, respectively. Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) (Gibco) was used as the secondary antibody for all immunoblots. Hybridization was visualized by immersing membranes in Luminol (Amersham Pharmacia) for 1 min and exposing them to radiographic film.

Y1 adrenal tumor cell assay. The Y1 adrenal tumor cell assay for LT activity was performed (47). Y1 mouse adrenal tumor cells (ATCC CCL-79) were exposed to serial dilutions of bacterial periplasmic extracts obtained by treatment with lysozyme (23). Purified CT (Sigma) and diluent were used as positive and negative controls, respectively. After exposure and incubation, cells were examined for rounding by phase-contrast microscopy.

GM_1 ELISA. The GM_1 enzyme-linked immunosorbent assay (ELISA) for LT was performed (44) with commercial GM_1 (Sigma), anti-CT rabbit serum (Sigma), and horseradish peroxidase-conjugated goat anti-rabbit IgG (Gibco). Assays were performed on serially diluted bacterial periplasmic extracts with purified CT (Sigma) and diluent as positive and negative controls, respectively. Plates were read spectrophotometrically at 480 nm.

Competitive EAST1 ELISA. The competitive EAST1 ELISA was performed as previously described (17). Bacterial cultures were grown in 10 ml of Luria-Bertani (LB) broth overnight at 37°C with aeration. Cells were harvested by centrifugation, resuspended in 5 ml of ice-cold 1× phosphate-buffered saline (PBS; pH 7.0) containing 10-μg ml^{-1} soybean trypsin inhibitor (Sigma) and 1 mM EDTA, and sonicated. Cell debris was removed by centrifugation at 10,000 × *g* for 20 min at 4°C, and the supernatants were used directly for EAST1 ELISA. Antisera to synthetic peptide containing the N-terminal 18 amino acids of EAST1 cross-linked to keyhole limpet hemocyanin were raised in rabbits by standard immunization methods (kindly provided by W. Fusco, University of Idaho, Moscow). Purified IgG antibodies were obtained from the antiserum by using a protein A column (Amersham Pharmacia) per the manufacturer's instructions. Wells of 96-well flat-bottom polyvinyl plates were coated with 100 μl of 1× PBS (pH 7.0) containing 5 ng of synthetic EAST1 peptide (the 18 N-terminal amino acids of EAST1 conjugated to ovalbumin). Coating solutions were incubated in the microtiter plates overnight at 37°C. Coating solutions were removed by tapping the plates dry, and 180 μl of blocking solution (1% ovalbumin in 1× PBS [pH 7.0]) was added to each well. After incubation for 1 h at 37°C, blocking solutions were removed and plates were washed three times with wash buffer (0.05% Tween 20 in 1× PBS [pH 7.0]) and tapped dry. Peptide standards (50 μl containing 10 to 100 ng per well) and test samples, diluted in ELISA dilution buffer (0.05% ovalbumin in 1× PBS [pH 7.0]), were mixed with 50 μl of rabbit anti-EAST1 serum (1:1,000) and applied to wells. Plates were incubated at 37°C for 2 h, with shaking. Peptide standards and unknown samples were assayed in triplicate. After incubation, samples were removed and wells were washed with wash buffer and tapped dry. One hundred microliters of goat anti-rabbit IgG labeled with horseradish peroxidase (1:10,000; Gibco BRL) was added to each well, and plates were incubated for 1 h at 37°C. After incubation, plates were washed three times with wash buffer and tapped dry. One hundred microliters of *O*-phenylenediamine substrate (1 mg ml^{-1}; Sigma) in 1× stable peroxide substrate buffer (Pierce) was added to each well and incubated at room temperature for 20 min. The color reaction was stopped by addition of 50 μl of 3 M o-phosphoric acid. Absorbance was measured at 480 nm with a plate reader (Sunrise, Tecan).

Construction of *eltAB* deletion and complemented mutants. Plasmid pMUN281 was constructed by removing a 910-bp SmaI-XbaI fragment ($eltAB_{171\text{-}1081}$) from pWD600 and replacing it with a 1,240-bp kanamycin resistance (Km^r) cassette (*aphA*) from pUC4K (59). This resulted in deletion of the terminal 692 bp of *eltA* and the first 220 bp of *eltB*. This *eltAB* region containing the Km^r cassette was cloned into the XbaI site of pCVD442 and transformed into S17-18λ*pir* to yield donor strain MUN283. Mating mixtures of MUN283 and WAM2317 were subcultured overnight on LB agar containing nalidixic acid (50 μg ml^{-1}) and kanamycin (50 μg ml^{-1}) (LB-NAL-KM). Transconjugant isolates on LB-NAL-KM plates were tested for reduced LT synthesis by GM_1 ELISA (44). Isolates with reduced LT synthesis were tested for sucrose sensitivity by plating on LB medium containing 5% sucrose. Isolates identified as sucrose sensitive were subcultured in LB broth without NaCl. Serial dilutions of these cultures were plated on LB agar containing 5% sucrose, nalidixic acid, kanamycin, and no NaCl (LB-NAL-KM-sucrose) and incubated overnight at 28°C. LB-NAL-KM-sucrose plates were screened for LT^- deletion mutants by colony blot hybridization by first probing with the *aphA* gene and then with the $eltAB_{171\text{-}1081}$ fragment. Colonies that hybridized with *aphA* but not the deletion site probe were streaked onto LB-NAL-KM-sucrose plates to confirm purity and appropriate antibiotic resistance patterns and were tested by PCR with the oligonucleotide primers LTABU and LTABL outflanking the deleted region. Transconjugants that had undergone a single-crossover event were identified by the production of both a 1,274-bp amplicon and a 1,604-bp amplicon, whereas those that had undergone a double-crossover event were identified by the production of only the larger product. The genomic DNA of strains that produced only the 1,604-bp product were digested with HindIII and XbaI and subjected to Southern blot analyses with *eltAB*, *aph*, and deletion site probes. Periplasmic extracts of strains that were *eltAB* and *aphA* probe positive and deletion site probe negative were tested for LT activity by GM_1 ELISA and Y1 adrenal cell and immunoblot assays. Strains that lacked detectable LT activity by all three methods were identified as LT^- mutants. For LT complementation, a PCR-amplified 1,274-bp *eltAB* fragment from WAM2317 was cloned into pMMB66, generating pMUN287. This plasmid was transformed into identified LT^- mutants by electroporation. Complemented strains were tested for LT expression by GM_1 ELISA and Y1 adrenal cell and immunoblot assays. All strains used for piglet inoculation experiments were tested for STb and EAST1expression by reverse transcription-PCR (RT-PCR), Northern blot analyses, and immunoblot analyses.

Gnotobiotic piglet experiments. A total of 20 gnotobiotic purebred Yorkshire piglets from two litters were derived by closed hysterotomy and reared in sterile isolator units according to standard procedures (32). Each litter was divided at random into five groups, with one group each reared in an individual isolator unit. When 1, 6, and 9 days old, swab samples from the nasal cavity and rectum were obtained from each piglet and cultured aerobically and anaerobically by routine methods to test for sterility. When the piglets were 7 days old, preinoculation blood samples were obtained for culture and serum assays. When 9 days old, each piglet was inoculated with 10^9 CFU of strain 2534-86, WAM2317, MUN285, MUN287, or G58-1 in 50 ml of sterile milk replacer. Each piglet was observed to consume the inoculum within 30 min. After inoculation, piglets were examined for decreased rectal temperature, anorexia, vomiting, diarrhea, depression, and a moribund condition. Piglets were examined and weighed prior to inoculation and after inoculation at 0, 6, and 12 h and then every 4 h until 96 h postinoculation, or when they became moribund, at which time they were subjected to blood sampling, euthanasia, and necropsy. A moribund condition was defined clinically as severe dehydration (evidenced by loss of skin turgidity and eyes sunken in the orbits), ≥23% weight loss, depression, hypothermia, and severe weakness. The rate of weight change for each piglet was calculated by dividing the difference between the prenecropsy and preinoculation body weights by the number of hours p.i. at necropsy. Experiments were preapproved by the University of Nebraska—Lincoln Institutional Animal Care and Use Committee.

Preinoculation and p.i. blood samples, collected aseptically, were transferred immediately to commercial brain heart infusion broth culture vials (Septi-Check; BBL Laboratories, Cockeysville, Md.) for bacterial culture. Aliquots were distributed into sodium heparin tubes for determination of the hematocrit (HCT) and serum separator tubes for determination of serum electrolytes and other analytes. Blood samples in serum separator tubes were allowed to clot for 20 min and then were centrifuged, and the serum was collected and frozen at −20°C until analyzed. Concentrations of urea nitrogen, creatinine, sodium (Na^+), potassium (K^+), chloride (Cl^-), total CO_2 (tCO_2), albumin, and total protein were measured in serum samples using a Vitros 500 automated chemistry analyzer (Ortho-Clinical Diagnostics). Rectal and nasal swab specimens to test for preinoculation sterility were cultured aerobically and anaerobically by standard procedures on sheep blood agar (5% sheep blood in Trypticase soy agar; Remel Labs, Lenexa, Kans.). Specimens of lung, liver, and ileum were collected aseptically at necropsy, weighed, ground with a tissue grinder in sterile PBS, and serially diluted in sterile peptone water. Serial dilutions were initially spread plated on LB medium and then replica plated to LB-NAL, LB-NAL-KM, LB-

NAL-KM-AP, and blood agar to determine the CFU of the respective inocula per gram of tissue. Bacterial isolates were identified by standard procedures, and the expected antibiotic resistance patterns of the various isolates were confirmed.

Histopathology, immunohistochemistry, and image analysis. At necropsy, the entire small intestine was dissected free from the mesentery and the length from the pyloric to the ileocecal valves was measured. Specimens were obtained from the following locations and fixed in 10% neutral buffered formalin for histopathological examination: duodenum (5 cm distal to the pyloric valve), jejunum-1 (one-third of the distance between the pyloric and ileocecal valves), jejunum-2 (half the distance between the pyloric and ileocecal valves, jejunum-3 (two-thirds of the distance between the pyloric and ileocecal valves), and ileum (5 cm proximal to the ileocecal valve). Specimens of stomach, duodenum, jejunum, ileum, spiral colon, mesenteric lymph node, lung, liver, spleen, kidney, and brain were also collected and fixed in formalin for histopathological examination. Formalin-fixed specimens were processed, embedded in paraffin, sectioned at 5 μm, and stained by hematoxylin and eosin by standard procedures. Tissue sections were examined by routine light microscopic examination. Five sections from each of five areas of small intestine (one duodenum, three jejunum, and one ileum) were graded for the severity of intestinal ischemia as previously described (6). Ischemia grades were from 0 to 5, with 0 representing normal intestine and 5 the most severe ischemia (6). The single most severe grade of ischemia seen among the 25 sections for each piglet was recorded as the ischemia score.

Additional sections of small intestinal specimens were cut from paraffin blocks and stained by an immunohistochemical procedure (45) using rabbit polyclonal antiserum raised against *E. coli* O8 or *E. coli* O101 (*E. coli* Reference Center, The Pennsylvania State University) as the primary antibody. This procedure was conducted on the ileal specimens of all piglets in both litters and on the duodenal and jejunal specimens of pigs from the second litter. Microscopic digitalized images of the mucosa of each of the five immunohistochemically stained intestinal sections on each area of intestine examined were collected by using a ×20 objective lens with a color digital camera. All images were recorded under the same microscope and camera settings, i.e., level of transmitted light, image size, contrast, brightness, and exposure time. Five frames of images on each section, each covering actual sample areas of 400 by 500 μm, were recorded with 300-μm intervals between collected images from each of the sections. All images were saved in the TIFF format and quantitatively analyzed with a SIS AnalySIS Opti imaging analysis program (Soft Imaging System, Inc., Lakewood, Colo.) for the areas covered by positive staining of *E. coli* O8$^+$ or O101$^+$ bacteria. After setting the color thresholds only for the areas labeled by specific antibodies in each image frame, the total area in each of the images was determined in square millimeters and pooled from five frames of each of the intestinal sections. Mean area measurements (square millimeters) of colonizing bacteria for each area of small intestine for each treatment group were reported as colonization scores.

Analysis of brush borders for susceptibility to adhesion of F4$^+$ *E. coli* and presence of IMTGP. The F4 adherence phenotype of each piglet (i.e., the susceptibility to F4ab, F4ac, and F4ad subtypes) was determined via brush border assays (2). Brush border specimens were subsequently tested for the presence of the F4ac receptor, IMTGP, by the biotinylated-adhesin overlay assay as previously described (20).

Statistical analyses. Data were analyzed by using the mixed procedure (SAS for Windows, version 8; SAS Institute, Cary, N.C.), and least-squares means for the different treatment groups were compared by using general *t* values. Calculated *P* values of <0.05 were considered significant.

RESULTS

Location of enterotoxin genes in wild-type strain 2534-86. Because previous studies have consistently shown that ETEC strains carry enterotoxin genes on large transmissible plasmids, we sought to determine whether this was the case with wild-type ETEC strain 2534-86. This strain was found to contain four plasmids with relative molecular sizes of approximately 130, 80, 60, and 50 kbp. Southern hybridization analyses determined that the genes coding for LT (*eltAB*) and STb (*estB*) were present on the 130-kbp plasmid, whereas the gene coding for EAST1 (*astA*) was located on the 80-kbp plasmid (data not shown). No copies of these genes were found on chromosomal DNA. These experiments were conducted subsequent to those involving allelic exchange, in which the *eltAB* genes detected in LT$^+$ strains originated from preparations of total genomic DNA.

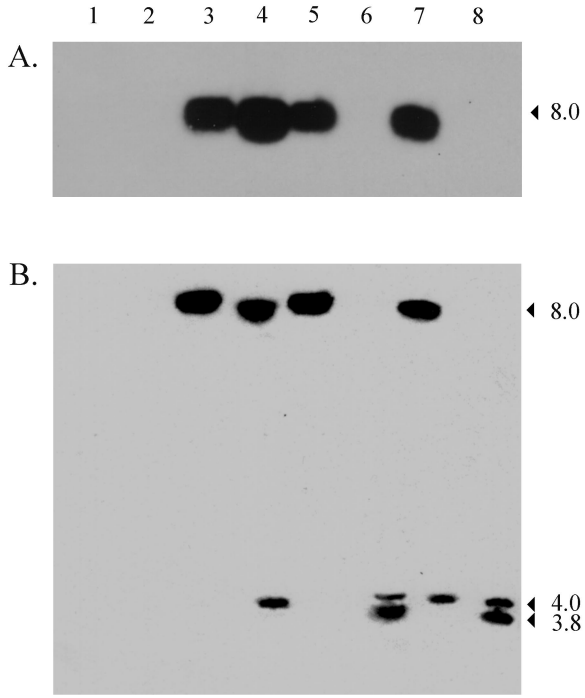

FIG. 1. Southern hybridization of XbaI digests of genomic DNA for (A) kanamycin resistance gene (*aphA*) and (B) *E. coli* heat-labile enterotoxin (LT) genes (*eltAB*). Lanes: 1, molecular size marker; 2, negative control DH5α; 3, LT$^-$ (Δ*eltAB*) mutant MUN284; 4, single-crossover mutant; 5, LT$^-$ (Δ*eltAB*) mutant MUN285; 6, recipient strain WAM2317; 7, single-crossover mutant; 8, LT$^+$ wild-type 2534-86. Horseradish peroxidase-labeled probes were used for *aphA* (450-bp internal PCR fragment) and *eltAB* (1,274-bp PCR product). Molecular sizes (kilobase pairs) are denoted by arrowheads to the right.

Construction of *eltAB* deletion and complemented mutants. LT$^-$ (Δ*eltAB*) mutants of strain WAM2317, containing a deletion of the terminal 692 bp of *eltA* and the first 220 bp of *eltB* and insertion of a 1,240-bp Km^r resistance cassette into the deletion site, were constructed by allelic exchange. Transconjugants that had undergone a single-crossover event were identified by the production of both a 1,274-bp amplicon and a 1,604-bp amplicon by PCR, whereas those that had undergone a double-crossover event were identified by the production of only the larger product (data not shown). Two strains that produced only the 1,604-bp product, designated MUN284 and MUN285, were further demonstrated by Southern blot analyses of HindIII and XbaI digests of their genomic DNA to be both *eltAB* and *aphA* probe positive and deletion site probe negative (Fig. 1). XbaI digests of the wild-type parent, 2534-86, and Nal^r recipient, WAM2317, resulted in two *eltAB* probe-positive fragments (4.0 and 3.8 kbp), due to the presence of an XbaI site internal to *eltA* at site 121. This XbaI site was deleted in the donor plasmid, resulting in a single 8.0-kbp fragment after complete allelic exchange. XbaI digestion of the single-crossover mutant DNA resulted in three fragments of approximately 8.0, 7.9, and 4.0 kbp.

Parent and LT$^-$ mutant strains were tested for LT activity by GM_1 ELISA and Y1 adrenal cell assays. Whereas periplasmic

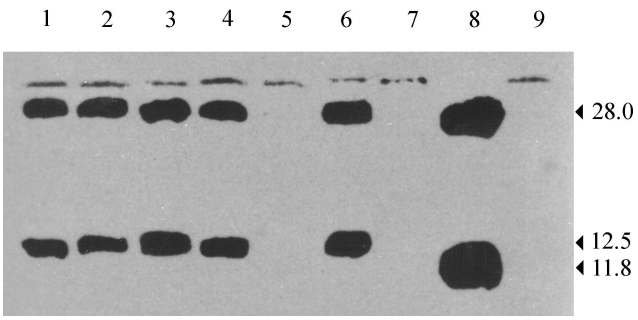

FIG. 2. Immunoblot of periplasmic protein extracts for *E. coli* heat-labile enterotoxin (LT). Lanes: 1, recipient strain WAM2317; 2, single-crossover mutant; 3, *eltAB* clone CB2091; 4, LT^+ wild-type 2534-86; 5, LT^- (Δ*eltAB*) mutant MUN284; 6, complemented mutant MUN287; 7, LT^- (Δ*eltAB*) mutant MUN285; 8, purified LT_h (Sigma); 9, negative control DH5α. Protein extracts were standardized for total protein concentration. The blot was probed sequentially with rabbit CT antiserum and horseradish peroxidase-conjugated goat anti-rabbit IgG. Detection was done by enhanced chemiluminescence. Numbers to the right denote molecular masses (kilodaltons).

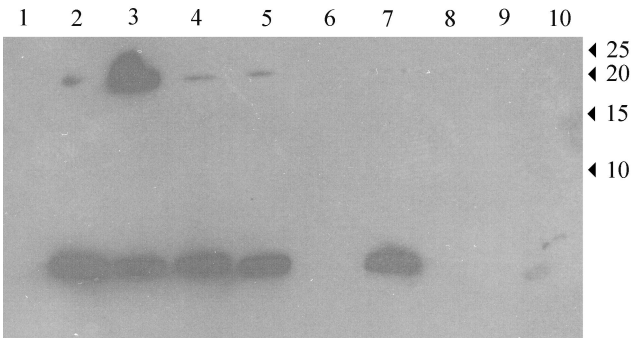

FIG. 3. Immunoblot of culture supernatants for STb. Lanes: 1, negative control G58-1; 2, LT^+ wild-type 2534-86; 3, recipient strain WAM2317; 4, LT^- (Δ*eltAB*) mutant MUN285; 5, complemented mutant MUN287; 6, negative control NADC 2290; 7, STb^+ clone NADC 2329; 8, molecular mass marker; 9, vector control NADC 2787; 10, STb^- mutant clone NADC 4924. Samples were standardized for total protein concentration. The blot was probed with rabbit anti-STb serum (12) and horseradish peroxidase-conjugated goat anti-rabbit IgG. Detection was done by enhanced chemiluminescence. Numbers to the right denote molecular masses (kilodaltons).

extracts of 2534-86 and WAM2317 contained marked LT activity as detected by these assays, no such activity was detected in extracts of strains MUN284 and MUN285 (data not shown). For LT complementation, a PCR-amplified 1,274-bp *eltAB* fragment from WAM2317 was cloned into pMMB66, generating pMUN287. This plasmid was transformed into MUN285, generating LT^+ strain MUN287. By immunoblot analysis using rabbit anti-CT serum, periplasmic extracts of 2534-86, WAM2317, and MUN287 contained 28- and 12.8-kDa proteins corresponding to the A and B subunits of LT, respectively (18), whereas these proteins were absent from periplasmic extracts of MUN284 and MUN285 (Fig. 2).

STb and EAST1 expression by LT^+ and LT^- ETEC strains. Strains 2534-86, WAM2317, MUN285, and MUN287, but not G58-1, were found to express both STb and EAST1 by RT-PCR (data not shown). Expression of STb by these strains was also shown by Northern (data not shown) and immunoblot analyses (Fig. 3). In immunoblots probed with rabbit anti-STb serum, all ETEC strains and STb^+ controls generated 5-kDa reaction products, consistent with STb (12). Reaction products of approximately 22 kDa were also produced by the ETEC strains and were interpreted to represent aggregates of STb monomers (12). Cytoplasmic preparations from the ETEC strains yielded activity in the EAST1 competitive ELISA comparable to that resulting from 50 ng (Fig. 4). Non-ETEC strain G58-1 and appropriate controls were negative for expression of STb in Northern (data not shown), RT-PCR (data not shown), and immunoblot (Fig. 3) assays and EAST1 in competitive ELISAs (Fig. 4).

The mean rate of weight loss in piglets inoculated with the LT^- mutant strain was significantly lower than that of piglets inoculated with the LT^+ Nal^r parent and complemented strains. We hypothesized that inactivation of LT would reduce the ability of ETEC bacteria to cause severe dehydrating diarrhea and postdiarrheal septicemia. To test this hypothesis, gnotobiotic piglets were inoculated with isogenic LT^- or LT^+ ETEC strains or the nonpathogenic control strain, G58-1, and examined for clinical evidence of diarrhea, weight loss, hypothermia, and a moribund state. Inoculated piglets were euthanized upon becoming moribund or at 96 h p.i. if a moribund condition had not yet developed. Twenty purebred Yorkshire piglets in two litters were used in the study. All piglets were either phenotype A (susceptible to F4ab, F4ac, or F4ad adherence) or B (susceptible to F4ab and F4ac adherence) in brush border assays. To further confirm susceptibility to $F4ac^+$-ETEC-mediated disease, all piglets were shown to express the F4ac fimbrial receptor, IMTGP, by the biotinylated-adhesin overlay assay (data not shown). All piglets inoculated with the LT^+ and LT^- ETEC strains developed diarrhea by 12 to 16 h p.i., and it persisted until they were euthanized. In contrast, all piglets inoculated with strain G58-1 remained clinically normal and maintained or gained weight over the 96-h-p.i. period.

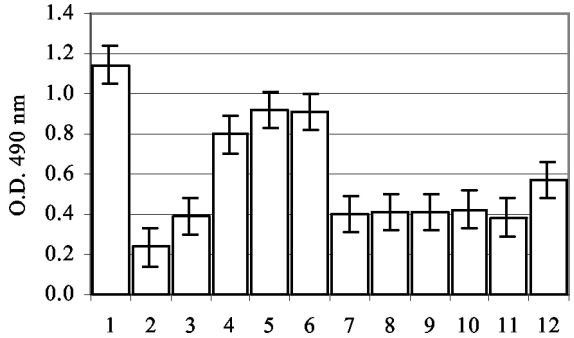

FIG. 4. Competitive ELISA to detect EAST1 expression. Bars: 1, blank; 2 to 4, EAST1 N-terminal synthetic peptide at 100 ng (bar 2), 50 ng (bar 3), and 10 ng (bar 4); 5, negative control DH5α; 6, negative control G58-1; wild-type 2534-86; 8, recipient strain WAM2317; 9, LT^- (Δ*eltAB*) mutant MUN285; 10, complemented mutant MUN287; 11, wild-type enteroaggregative *E. coli* strain 17-2; 12, $EAST1^+$ clone BF-3. Optical densities (O.D.) at 490 nm are averages from three independent assays. Cytoplasmic protein samples of each strain were run in triplicate in each assay. Protein extracts were standardized for total protein concentration. EAST1 N-terminal synthetic peptide, prototype enteroaggregative *E. coli* strain 17-2, and *astA* clone BF-3 were used as $EAST1^+$ controls.

Fifteen of 18 (83%) piglets inoculated with ETEC strains (LT$^-$ and LT$^+$ groups combined) developed severe weight loss and became moribund. At the time of euthanasia, these piglets had a mean change in body weight (postinoculation minus preinoculation) of $-27.2\% \pm 2.5\%$, and a mean change in rectal temperature of $-3.9°C$ ($P < 0.0001$). Sixty percent of piglets inoculated with the Δ*eltAB* mutant became moribund, compared to 100% in the LT$^+$ Nal^r parent and complemented mutant groups, 80% in the wild-type group, and 0% in the nonpathogenic control group. Because piglets were allowed to reach a moribund condition prior to euthanasia, weight loss was expressed on a per-hour basis prior to statistical analysis. The mean rate of weight loss of piglets inoculated with the Δ*eltAB* mutant was 67% less than that of piglets inoculated with the LT$^+$ Nal^r parent strain ($P = 0.0324$; Fig. 5A). In contrast, the mean rate of weight loss of the piglets inoculated with the complemented mutant strain was 30% greater than that of piglets inoculated with the LT$^+$ parent strain ($P < 0.0010$; Fig. 5A).

The rate of development of dehydration in piglets inoculated with the LT$^-$ mutant strain was significantly lower than that of piglets inoculated with the LT$^+$ ETEC strains. To test for the effects of LT inactivation on development of dehydration, HCT, total serum protein, blood urea nitrogen, and serum creatinine concentrations were determined. Relative increases in the concentration of each analyte were expected to occur proportionately with the degree of dehydration as a result of water loss (20). The mean p.i. concentrations at the time of euthanasia and the mean change in concentration (p.i. minus preinoculation) of these analytes among treatment groups were compared. The mean p.i. HCT of the Δ*eltAB* mutant group ($24.8\% \pm 3.9\%$) was significantly less than that of the LT$^+$ Nal^r parent strain ($34.9\% \pm 3.4\%$, $P = 0.0420$) and complemented mutant groups ($34.4\% \pm 3.0\%$, $P = 0.0384$). Similarly, the mean change in HCT of the Δ*eltAB* mutant group was significantly less than those of the LT$^+$ Nal^r parent strain ($P = 0.0002$), wild-type ($P = 0.0106$), and complemented mutant ($P = 0.0028$) groups (Fig. 5B). A trend for a lower mean change in creatinine was detected in the *eltAB* mutant group, in contrast to the LT$^+$ groups (data not shown).

Piglets inoculated with the LT$^-$ mutant strain had significantly higher serum bicarbonate and lower anion gap concentrations than those of piglets inoculated with the LT$^+$ ETEC strains. We hypothesized that LT inactivation would reduce the severity of bicarbonate loss and dehydration, thereby mitigating the increase in anion gap associated with severe diarrhea. To address this hypothesis, the p.i. serum bicarbonate (HCO_3^-; represented by the tCO_2) and anion gap [$(Na^+ + K^+) - (HCO_3^- + Cl^-)$] concentrations (millimoles per liter), and the changes in these analytes (p.i. − preinoculation) among LT$^+$ and LT$^-$ ETEC and control groups were compared. The mean p.i. HCO_3^- of the Δ*eltAB* mutant group (20.3 ± 2.3 mmol liter^{-1}) was higher than that of the LT$^+$ Nal^r parent strain group (13.2 ± 2.7 mmol liter^{-1}, $P = 0.0666$). In addition, the mean changes in HCO_3^- (Fig. 5C) and anion gap (data not shown) of the Δ*eltAB* mutant group were significantly lower than those of the LT$^+$ Nal^r parent strain group ($P = 0.0290$ and $P = 0.0121$, respectively).

Piglets inoculated with the LT$^-$ mutant strain had significantly lower serum potassium and less increase in chloride concentrations compared to those of piglets inoculated with the LT$^+$ ETEC strains. To determine the effect of LT inactivation on the development of electrolyte imbalances in inoculated piglets, the mean p.i. and mean changes in serum sodium (Na^+), potassium (K^+), and chloride (Cl^-) concentrations (millimoles per liter) among LT$^+$ and LT$^-$ ETEC and control groups were compared. We hypothesized that piglets inoculated with the LT$^-$ ETEC strain, by comparison to those inoculated with LT$^+$ strains, would have lower mean p.i. and mean changes in serum K^+ and Cl^- concentrations, because of less bicarbonate loss and therefore less acidosis and dehydration. No significant differences in the mean p.i. Na^+ or mean change in Na^+ concentrations among the different ETEC and control groups were detected ($P > 0.10$; data not shown). However, the mean p.i. K^+ (3.4 ± 0.4 mmol liter^{-1}) and Cl^- (98.8 ± 3.7 mmol liter^{-1}) concentrations in the Δ*eltAB* mutant group were significantly less than those of the LT$^+$ Nal^r parent strain (4.9 ± 0.5 mmol liter^{-1}, $P = 0.0303$; and 114.7 ± 4.4 mmol liter^{-1}, $P = 0.0167$, respectively) and complemented mutant (5.4 ± 0.4 mmol liter^{-1}, $P = 0.0044$; and 109.7 ± 3.9 mmol liter^{-1}, $P = 0.0634$, respectively) groups. Also, the mean change in K^+ and Cl^- concentrations in the Δ*eltAB* mutant group were significantly less than those of the LT$^+$ Nal^r parent ($P = 0.0118$ and $P = 0.0154$, respectively, data not shown). Similarly, the mean change in K^+ concentration in the Δ*eltAB* mutant group was significantly less than that of the complemented strain group ($P = 0.0035$; data not shown).

The mean level of bacterial colonization in the jejunum and ileum in piglets inoculated with the LT$^-$ mutant strain was significantly reduced compared to that of piglets inoculated with the LT$^+$ ETEC strains. The ability of the different isogenic LT$^+$ and LT$^-$ ETEC strains to colonize the small intestine was determined on ileal tissues by plate counts and by image analysis of tissue sections of duodenum, jejunum, and ileum stained immunohistochemically for *E. coli* O8$^+$ antigen. The mean ileal plate count ($\log_{10}$ CFU per gram) of piglets inoculated with the Δ*eltAB* mutant strain (9.3 ± 0.7 $\log_{10}$ CFU g^{-1}) was 2.2 to 2.9 logs lower than that of piglets inoculated with LT$^+$ strains (2534-86, 11.5 ± 0.7 $\log_{10}$ CFU g^{-1}, $P = 0.0446$; WAM2317, 12.0 ± 0.9 $\log_{10}$ CFU g^{-1}, $P = 0.0329$; and MUN287, 12.0 ± 0.7 $\log_{10}$ CFU g^{-1}, $P = 0.0105$]). In contrast, the mean ileal plate count of the Δ*eltAB* mutant strain group was nearly identical to that of the nonpathogenic control group (9.4 ± 1.2 $\log_{10}$ CFU g^{-1}, $P = 0.9288$). Similarly, the mean ileal colonization score (square millimeters) of the Δ*eltAB* mutant strain group (both litters combined) was significantly reduced compared to the LT$^+$ Nal^r parent group ($P = 0.0484$), and the reduction compared to the complemented group approached statistical significance ($P = 0.0511$; Fig. 5D). The mean jejunal colonization score of the Δ*eltAB* mutant strain group in two of three areas examined was also significantly reduced compared to that of the LT$^+$ Nal^r parent group (Table 2). In contrast, colonization was restored in the complemented mutant group ($P > 0.10$).

Piglets inoculated with the LT$^-$ mutant strain had reduced incidences of intestinal ischemia, bacterial translocation, and secondary septicemia compared to those piglets inoculated with LT$^+$ ETEC strains. We hypothesized that LT inactivation would reduce the incidence of postdiarrheal hypovolemic shock, bacterial translocation, and secondary septicemia. Pres-

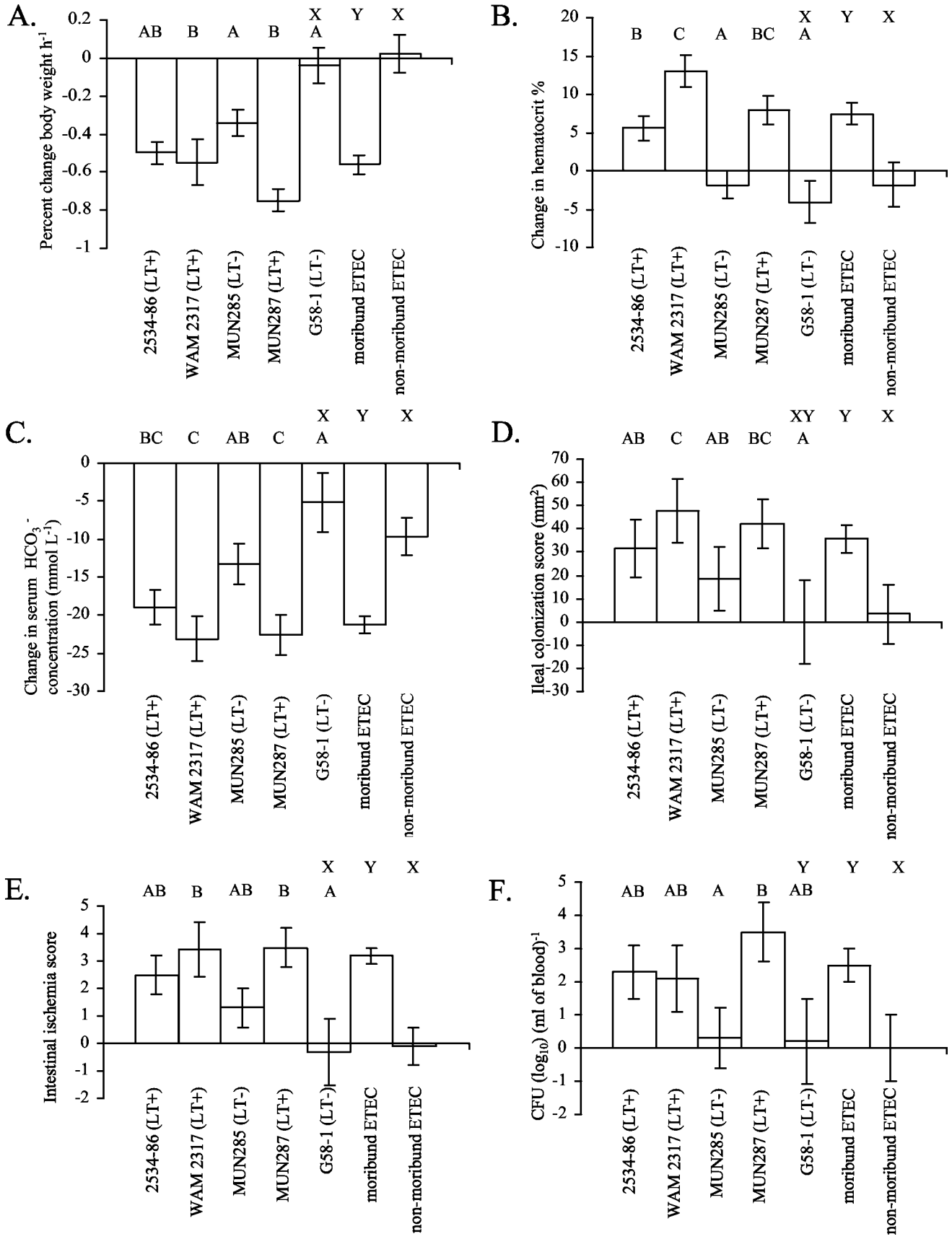
A.
Percent change body weight h-1
AB B A B X A Y X
2534-86 (LT+)
WAM 2317 (LT+)
MUN285 (LT-)
MUN287 (LT+)
G58-1 (LT-)
moribund ETEC
non-moribund ETEC
B.
Change in hematocrit %
B C A BC X A Y X
C.
Change in serum HCO3- concentration (mmol L-1)
BC C AB C X A Y X
D.
Ileal colonization score (mm2)
AB C AB BC XY A Y X
E.
Intestinal ischemia score
AB B AB B X A Y X
F.
CFU (log10) (ml of blood)-1
AB AB A B Y AB Y X

TABLE 2. Mean bacterial colonization scores in small intestine[a]

Area of small intestine[b]	Mean distance distal to pyloric valve (cm)	Bacterial colonization score (mm^2)		
		LT$^+$ Nal^r parent WAM2317 (n = 1)	LT$^-$ mutant MUN285 (n = 3)	LT$^+$ complemented mutant MUN287 (n = 3)
Duodenum	5 ± 0	7.83 ± 3.50	0.35 ± 2.02	7.06 ± 2.02
Jejunum-1	126 ± 13	9.00 ± 2.41	0.51 ± 1.39*	8.87 ± 1.39
Jejunum-2	188 ± 19	21.24 ± 4.02	0.67 ± 2.32*	8.63 ± 2.32
Jejunum-3	251 ± 26	15.83 ± 6.87	0.73 ± 3.97	14.53 ± 3.97
Ileum	371 ± 31	87.12 ± 22.38	10.76 ± 12.92*	41.57 ± 12.91

[a] Data reported are least-squares means ± standard errors. Asterisks denote statistically significant difference ($P < 0.05$) between LT mutant strain MUN285 and LT$^+$ Nal^r parent strain WAM2317. Data were obtained from a second litter of pigs.

[b] Specimens were obtained from the following locations: duodenum (5 cm distal to the pyloric valve); jejunum-1 (one-third of the distance from the pyloric to the ileocecal valve), jejunum-2 (one-half the distance from the pyloric to the ileocecal valve), jejunum-3 (two-thirds of the distance from the pyloric to the ileocecal valve), and ileum (5 cm proximal to the ileocecal valve). Specimens were fixed in 10% neutral buffered formalin, processed, sectioned, and stained by an immunohistochemical procedure (45) to detect *E. coli* O8$^+$ bacteria. Microscopic digitalized images of the mucosa were recorded and quantitatively analyzed for the areas containing positive staining for *E. coli* O8$^+$ bacteria.

ence of hypovolemic shock was based on detection of lesions of ischemic bowel necrosis in conjunction with severe dehydration, weight loss, and hypothermia. Regarding incidence data, the numbers of piglets per treatment group were too small for valid chi square tests; hence, only summary statistics are reported. Overall, the incidence of a moribund condition, intestinal ischemia lesions, and positive blood culture results for O8$^+$ *E. coli* were highly correlated. Lesions of intestinal ischemia and positive blood culture results were detected in 15 of 15 moribund piglets (100%), 0 of 3 nonmoribund ETEC-inoculated piglets (0%), and 0 of 2 controls (0%). The Δ*eltAB* mutant strain group showed a trend for a lower mean intestinal ischemia score, whereas the mean ischemia scores of the complemented mutant and LT$^+$ Nal^r parent groups were nearly identical (P = 0.9442; Fig. 5E). Only one of three ETEC-inoculated piglets that did not develop intestinal ischemia (nonmoribund) had detectable O8$^+$ bacteria in their intestinal blood and/or lymphatic vessels. This piglet was inoculated with the wild-type strain but lost no weight during the study and was blood culture negative for *E. coli*. In contrast, all moribund piglets and all LT$^+$ ETEC-inoculated piglets, but only 60% of the *eltAB* mutant strain piglets had O8 bacteria in their intestinal blood and/or lymphatic vessels. The incidence of septicemia in the Δ*eltAB* mutant strain was 20%, compared with 100% for the LT$^+$ Nal^r parent group. In addition, the Δ*eltAB* mutant strain group had a trend for a lower mean blood bacterial count compared to that of the LT$^+$ Nal^r parent group and was significantly lower than that of the complemented mutant group (P = 0.0275; Fig. 5F).

Gross and microscopic postmortem lesions in moribund piglets among LT$^+$ and LT$^-$ ETEC groups were not different. All had platelet or fibrin-platelet thrombi in small blood vessels of multiple organs; these lesions were indicative of disseminated intravascular coagulation (DIC). The stomach was the organ most consistently affected and had characteristic hemorrhagic mucosal infarcts in association with thrombosed mucosal and submucosal blood vessels. Grossly, these infarcts were uni- or bilateral and adjacent to the midline of the greater curvature. In every case, in addition to gastric thrombi, piglets with disseminated intravascular coagulation had thrombi in one or more other organs, including the liver, small and large intestines, lung, brain, and/or spleen. *E. coli* O8$^+$ bacteria were often noted to have heavily colonized the exposed basement membranes of ischemic villi. Subjacent villous capillaries also were noted to contain these bacteria, suggesting bacterial entry occurred across the exposed basement membranes following the development of intestinal ischemia.

DISCUSSION

This study, using isogenic LT$^+$ and LT$^-$ mutants, demonstrates the importance of LT as a virulence factor in ETEC infection and suggests the effects of LT are additive with those of STb and EAST1 in strains that produce all three toxins. Heretofore, the only studies that have directly tested the significance of LT were those based on the use of crude whole-cell lysates and broth culture supernatant fluids prepared from LT$^+$ or LT$^+$ STb$^+$ ETEC strains (21, 25, 37, 54). In these studies, lysates or supernatant fluids were injected into ligated intestinal segments of piglets or rabbits or were given intragastrically to piglets (20, 25, 26, 37, 54). Significant fluid accumulation occurred in ligated intestinal loops of piglets and rabbits (21, 37, 54); whole-cell lysates administered intragastrically to 1- to 8-day-old piglets caused up to 18% loss of body weight within 6 h after the onset of diarrhea and a 53% mortality rate (25). In these previous studies, the basis for attributing the

FIG. 5. Effects of inoculation of gnotobiotic piglets with isogenic LT$^+$ and LT$^-$ ETEC strains. Results are presented as the least-squares (LS) means ± standard error. Superscripts A to C refer to statistical comparisons of LS means of different treatment groups (inoculation with different bacterial strains), and X to Y refer to statistical comparisons of total moribund ETEC, total nonmoribund ETEC, and G58-1 (control) groups, respectively. Total moribund ETEC and total nonmoribund ETEC include all data combined, respectively, for moribund and nonmoribund animals inoculated with ETEC strains. LS means with unlike superscripts (e.g., A versus B or X versus Y) are significantly different based on the general t values ($P < 0.05$). Blood and serum analyte concentrations were determined on the p.i. specimen obtained immediately prior to euthanasia; differences between the p.i. and preinoculation values were reported as the change in concentration. (A) Rate of change in body weight. (B) Change in HCT. (C) Change in serum bicarbonate (HCO_3^-) concentration. (D) Ileal colonization score. (E) Intestinal ischemia score. (F) Blood bacterial concentration.

effects to LT was that heat treatment of whole-cell lysates (the main source of LT) completely abrogated enterotoxic activity (25). A strain used in that study (viz., strain 263) also produced a heat-stable enterotoxin (46), which explains why broth culture supernatant fluids, later shown to predominantly contain STb (12), only cause transient diarrhea (25). Antiserum to whole-cell lysates protected gnotobiotic piglets against the heat-labile enterotoxic activity (26). Although these studies strongly suggested that LT plays an important role in disease, this hypothesis had not been tested with isogenic LT^+ and LT^- ETEC strains or purified toxin.

In piglets inoculated with either LT^+ or LT^- strains, hypovolemic shock resulted from severe diarrheagenic water loss. Ischemic bowel necrosis, a lesion consistent with hypovolemic shock (6, 22), predisposed the piglets to septicemia. Sloughing of enterocytes from ischemic intestinal villi resulted in villous atrophy, a lesion previously reported to occur in severe ETEC infections of swine (35). Large numbers of ETEC bacteria adhered directly to necrotic villous epithelial basement membranes and capillary walls and were also found locally in subjacent villous capillaries. Hence, bacteria entered the bloodstream by this route. Endotoxemia and DIC may exacerbate the effects of hypovolemic shock in the development of ischemic bowel necrosis (8). Interestingly, *E. coli* O8:K87 (the serotype of the isogenic ETEC strains in this study) is typically one of few serotypes of *E. coli* that is serum resistant (56). Serum resistance assays conducted in our laboratory with the wild-type parent strain used in this study (2534-86) confirmed that this organism is resistant to gnotobiotic piglet serum (data not shown). However, the organism is susceptible to killing by serum containing antibodies to K87, and specific removal of these antibodies mitigates killing activity (N. M. Clark and R. A. Moxley, 83rd Conf. Res. Work Anim. Dis., abstr. 70P, 2002). The resistance of this organism to nonimmune serum probably enhanced survival in the blood and development of septicemia and DIC. These findings are consistent with observations of natural disease in conventional piglets: ETEC serotype O8:K87, referred to as G7 in the older literature (49), in particular is a significant cause of bacteremia in piglets less than 1 week old (49) and endotoxic shock manifested at necropsy by hemorrhagic gastroenteritis in pre- and postweaned pigs (40).

Serum chemistry results of piglets with hypovolemic shock in this study resembled those of human patients with cholera gravis (61). Evidence of dehydration was supported by the clinical appearance of the piglets (especially severe weight loss), coupled with increased HCT and a tendency toward increased total protein and creatinine concentrations (19). Albumin was measured, but some piglets had baseline albumin concentrations below the analytical linearity of the assay (1 g dl^{-1}), so numerical comparisons could not be made. While dehydration is expected to result in increased serum urea nitrogen and creatinine concentrations due to decreased renal glomerular filtration, and in the case of urea nitrogen, increased renal tubular resorption, increasing serum creatinine concentrations may have been lessened by improving renal function. Increases in serum urea nitrogen concentrations may have also been masked by a decreased intake, coupled with increased intestinal excretion of protein.

The greater drop in tCO_2 (bicarbonate) concentrations in piglets inoculated with LT^+ ETEC strains was evidence of metabolic acidosis. This was presumably due mostly to intestinal HCO_3^- loss, as increased crypt epithelial Cl^- secretion caused by LT may lead to villus Cl^-/HCO_3^- exchange (i.e., Cl^- resorption and HCO_3^- secretion). To maintain electroneutrality, net losses of HCO_3^- are often accompanied by decreased excretion of Cl^-, the body's most abundant extracellular anion, leading to a hyperchloremic metabolic acidosis. The presence of an increased anion gap supports the presence of unmeasured anions. Given the hypovolemia, these were likely lactic acid from increased anaerobic metabolism and possibly anions that were retained due to some decrease in glomerular filtration rate (e.g., phosphates). Albumin was also likely increased because of dehydration, unless there was intestinal loss secondary to devitalized intestinal tissue. The increased serum K^+ concentrations occurring with WAM2317 and MUN287 probably resulted largely from exchange of intracellular K^+ for extracellular H^+. Increased plasma K^+, increased plasma osmolality, decreased plasma HCO_3^-, and decreased plasma Na^+ were detected in piglets infected with ETEC in a previous study (35). In that study, data were compiled from moribund piglets inoculated with different ETEC strains; the type of enterotoxins produced by these strains was not reported (35). However, strain 263 was one of the strains used; as noted above, this strain was later shown to produce LT and ST, but the type of ST was not identified (46). One difference in the present study was that a mean decrease in serum Na^+ concentration was not detected in piglets inoculated with ETEC strains.

Inactivation of LT did not appear to change the outcome of the serum chemistry results, which suggests that the other enterotoxins produced by this strain had effects on water and electrolyte losses similar to those caused by LT. Similar clinical pathology test results among piglets inoculated with LT^+ STb^+ $EAST1^+$ and LT^- STb^+ $EAST1^+$ strains might be predicted based on the current knowledge of the effects of each toxin. LT activates adenylate cyclase in enterocytes, which causes increased intracellular concentrations of cyclic AMP (cAMP) (42). Increased cAMP concentration results in stimulation of Cl^- secretion from crypt epithelial cells and inhibition of NaCl absorption by villous enterocytes with resultant osmotic diarrhea (42). STa activates guanylate cyclase in enterocytes, resulting in increased intracellular concentrations of cGMP, Cl^- secretion, and inhibition of NaCl absorption in the small intestine (42, 52). STb stimulates secretion of HCO_3^- from enterocytes, which causes its accumulation, along with increased concentrations of Na^+ and Cl^-, in the intestinal lumen (11, 63). EAST1 has significant homology with the enterotoxic domain of STa and with guanylin, a mammalian analog of STa (29, 50). EAST1 elicits enterotoxic activity via stimulation of increased cGMP concentrations within enterocytes (29, 50). EAST1 has not yet been purified to homogeneity (29); however, synthetic EAST1 peptide was found to induce a significant rise in short-circuit current in Ussing chambers that was similar, but not identical to the electrogenic response evoked by STa (50). The effects of EAST1 on induction of electrolyte loss from the intestine have not yet been determined. In addition to the direct effects of different enterotoxins on the intestinal epithelium, recent studies suggest that many of these toxins, including LT, cause diarrhea through their effects on

the enteric nervous system (15, 27, 39). Vasoactive intestinal polypeptide is thought to be an important mediator of the enteric nervous system effects induced by LT, CT, and STa (15, 27, 39).

The importance of the different enterotoxins in ETEC infections of swine must be explored in light of the fimbrial type, fimbrial receptor status, and host age. Heretofore, the only study that had been done with isogenic *E. coli* enterotoxin-positive and -negative strains was in neonatal piglets inoculated when <8 h old and involved a comparison of the pathogenicity of F41$^+$ STb$^+$ and STb$^-$ strains (4). In that study, expression of STb did not contribute significantly to weight loss in the neonatal piglet (4). The lack of effect of STb in the neonatal piglet has been attributed to the relatively high level of trypsin activity in the jejunum at this age, which inactivates the toxin (64). The only study in which the pathogenic effects of known LT$^+$ and LT$^-$ ETEC have been compared in swine utilized wild-type isolates of different fimbrial types (F4, F5, or F6) that produced STa in neonatal piglets and in which the fimbrial receptor status was not determined (34). In that study, the mean percent weight loss of piglets inoculated with LT$^-$ STa$^+$ ETEC strains was 15%; most (45 of 59) of the strains studied were F6$^+$, only one was F5$^+$, and the rest produced no detectable fimbria. In contrast, the mean weight loss of piglets inoculated with one of two different LT$^+$ STa$^+$ ETEC strains (one F4$^+$ and the other producing no detectable fimbria) was only 3%. Presumably, the variation in fimbrial production and piglet susceptibility prevented detection of an additive effect of LT production by the challenge strains. With new evidence that F4$^+$ porcine ETEC isolates commonly carry the EAST1 gene (*astA*), the relative importance and roles of this enterotoxin must also be addressed.

A common explanation of the benefit of a diarrheagenic toxin to the respective bacterium is to flush progeny organisms into the environment, thereby improving the chances of spread to new hosts and persistence of the species (58). This explanation is reasonable; however, it is possible these toxins provide additional benefits to the bacterium. Our finding that piglets inoculated with the LT$^-$ mutant strain had significantly reduced colonization in the jejunum and ileum suggests that LT activity may enhance bacterial colonization of the small intestine, and similar results have been reported for CT in a rabbit model (43). Nontoxigenic A$^-$ B$^-$ and A$^-$ B$^+$ isogenic mutant strains of *V. cholerae* colonized less efficiently than the fully toxinogenic parent strain, and colonization by an A$^-$ B$^-$ mutant was increased to the level of the parent by coadministration of CT with the inoculum (43). These results were interpreted as evidence that CT promotes mucosal colonization by *V. cholerae*, not by interaction of the B subunit with its membrane receptor, but by a pharmacologic effect on the mucosa (43). Based on the fact that CT and LT have similar biological effects, the finding of reduced intestinal colonization with CT$^-$ and LT$^-$ mutants in these different studies raises an intriguing question of whether the two toxins may share certain direct or indirect effects that enhance bacterial colonization of the small intestine.

LT could potentially enhance bacterial colonization of the small intestine by several mechanisms. It could potentially act as an adhesin, e.g., in a manner similar to pertussis toxin. Pertussis toxin, which shares features with CT and LT in that it is A-B in structure and mediates ADP-ribosylation of a G protein, causing elevated intracellular levels of cAMP, acts as an adhesin of *Bordetella pertussis* for human ciliated respiratory epithelial cells (60). However, since CT does not enhance mucosal colonization of *V. cholerae* by interaction of the B subunit with its receptor, the potential for LT acting as an adhesin would seem less likely. As proposed for CT (43), LT might somehow favor bacterial colonization and growth via induced alterations in intestinal function: e.g., altered motility or enhanced secretion of water, electrolytes, mucus, and enteric hormones. Alternative hypotheses are that LT activity may stimulate expression of a colonization factor, such as the F4 fimbriae, or may expose fimbrial receptors because of hydration of the microvilli and thinning of the glycocalyx. The F4 fimbriae appear to be associated primarily with strains that also produce LT, and it would not be surprising if there were a functional relationship between them.

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